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Effects of environmental concentrations of the fragrance amyl salicylate on the mediterranean mussel *Mytilus galloprovincialis*☆

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ABSTRACT

Amyl salicylate (AS) is a fragrance massively used as a personal care product and following the discharged in wastewaters may end up in the aquatic environment representing a potential threat for the ecosystem and living organisms. AS was recently detected in water of the Venice Lagoon, a vulnerable area continuously subjected to the income of anthropogenic chemicals. The lagoon is a relevant area for mollusc farming, including the Mediterranean mussels (*Mytilus galloprovincialis*) having an important economic and ecological role. Despite high levels of AS occurred in water of the Lagoon of Venice, no studies investigated the possible consequences of AS exposures on species inhabiting this ecosystem to date. For the first time, we applied a multidisciplinary approach to investigate the potential effects of the fragrance AS on Mediterranean mussels. To reach such a goal, bioaccumulation, cellular, biochemical, and molecular analyses (RNA-seq and microbiota characterization) were measured in mussels treated for 7 and 14 days with different AS Venice lagoon environmental levels (0.1 and 0.5 μg L^{-1}). Despite chemical investigations suggested low AS bioaccumulation capability, cellular and molecular analyses highlighted the disruption of several key cellular processes after the prolonged exposures to the high AS concentration. Among them, potential immunotoxicity and changes in transcriptional regulation of pathways involved in energy metabolism, stress response, apoptosis and cell death regulations have been observed. Conversely, exposure to the low AS concentration demonstrated weak transcriptional changes and transient increased representation of opportunistic pathogens, as *Arcobacter* genus and *Vibrio aestuarianus*. Summarizing, this study provides the first overview on the effects of AS on one of the most widely farmed mollusk species.

1. Introduction

The Mediterranean mussel (*Mytilus galloprovincialis*) is an ecological and economic important edible bivalve species widely distributed in the Mediterranean Sea and in lagoon waters, in natural beds and farming areas. Among European countries, Italy represents one of the main leader countries in mussel production, second only to Spain (64,000 tonnes/year) (European Market Observatory for Fisheries and Aquaculture Products, 2019), with the Lagoon of Venice representing one of the most important farming areas. This productive area is widely affected by both urban and industrial pollution mainly due to the domestic sewage from Venice, the agricultural drainage from inland and the activities performed in Porto Marghera industrial area. Accordingly, the Venice lagoon is under constant surveillance to identify and characterize environmental criticalities representing potential threats to the ecosystem and human health. Among the most important ongoing research activities, the characterization of emerging contaminants in the water column and sediments led to the detection of high concentrations of fragrances including salicylates (Vecchiato et al., 2016).

Fragrances are "chemical mixtures obtained by natural aromatic raw

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and/or synthetic materials that smell of characteristic odor" as defined by the International Fragrance Association (IFRA). They are ingredients in several daily life Personal Care Products (PCPs), as perfumes, cosmetics and toiletries (Bauer et al., 1997). Fragrances, and PCPs more in general, are widely produced all over the world (Liu and Wong, 2013) and following their extensive application, they (and their metabolic conjugates) are discharged in wastewaters (Patel et al., 2009). Going through sewage treatment plants (STPs) they may end up into seawater (Díaz-Cruz et al., 2009), assuming the role of potential threat for aquatic species mainly due to their persistence and/or their incessant inputs (Casatta et al., 2015; Chase et al., 2012). While this class of pollutants is not currently included in EU routine monitoring programs (Patel et al., 2009), the scientific community has classified fragrances as Contaminants of Emerging Concern (CECs) (Tang et al., 2017), implying the need of further studies to fill the critical ecotoxicological knowledge gap on their potential environmental impacts (Chase et al., 2012). Among others, recent studies highlighted disfunction in larval development in the copepod *Acartia tonsa* by Salycilates (Picone et al., 2021) and effects in larval development of *M. galloprovincialis* and *Paracentrus lividus* following exposure to Galoxolide and Tonalide (Ehiguese et al., 2021). In earthworms, the same chemicals led to physiological responses and transcriptional changes in antioxidant genes (Chen et al., 2011), whereas it caused oxidative and genetic damage in the freshwater mussel *Dreissena polymorpha* (Parolini et al., 2015). In addition, effects of musks in reproduction and early life-stage survival in zebrafish (Carlsson et al., 2000; Carlsson and Norrgren, 2004) and in the freshwater mussel *Lampsilis cardium* (Gooding et al., 2006) were also observed. However, studies on the marine ecotoxicity are still scarce, underlining thus the lack of an effective assessment of the risk of these substances (Ehiguese et al., 2020).

Amyl salicylate (AS), ($C_{12}H_{16}O_3$, IUPAC name: pentyl 2-hydroxybenzoate) (CAS No, 2050-08-0) is a chemical compound belonging to the salicylate family that is largely used in perfumery and in other personal care products because of its organoleptic properties and the low cost (under $5/kg) (Gaudin, 2014). Its global use has increased over the last two decades, amounting around 100–1000 metric tons per annum (Belsito et al., 2007; Lapczynski et al., 2007). Because of the extensive usage, the fate of this compound might reflect the fate of other fragrances and PCPs. Vecchiato and colleagues described AS as one of the most widespread fragrances, from the open sea areas of the Mediterranean offshore waters (detected concentrations between 0.007 and 0.06 μg L^{-1}) to the innermost canals of Venice where it reaches levels up to 6 μg L^{-1} (Vecchiato et al., 2016, 2018) and the Antarctic Sea (Vecchiato et al., 2017). Moreover, an ice core from Elbrus, Caucasus, showed that during the 20th century the industrial production let to a 20-fold increase of its deposition fluxes (Vecchiato et al., 2020). Despite AS has been recently classified as very toxic to aquatic life by ECHA in REACH registrations (ECHA, 2020), few studies have explored the potential accumulation and effects of this compound for aquatic fauna to date (Picone et al., 2021; Fabrello et al., 2021).

In the present study, we investigated the effects of AS on the Mediterranean mussel *Mytilus galloprovincialis* following exposure of bivalves to two concentrations of AS commonly found in seawater of the Lagoon of Venice. A multidisciplinary approach based on chemical (bioaccumulation), cellular, biochemical, and molecular analyses (microbiota and transcriptional profiling) was adopted to assess the effects of this compound on one of the most widely farmed mollusk species.

2. Material and methods

2.1. Experimental design

Previous research programs performed in 2019–2020 identified AS (CAS no.: 2050-08-0) as one of the most represented fragrance ingredients in the water column of the Venice lagoon. In our study, a total of 270 adults of *M. galloprovincialis* (average length 53.8 ± 5.1 mm) were sampled in June, during the resting phase of mussels (Da Ros et al., 1985) from a clean farming area located in the south of the Venice Lagoon (median AS concentrations = 0.0075 μg L^{-1}; unpublished data). Mussels were left to acclimate with aerated seawater (salinity of 35 ± 1, temperature of 18 ± 0.5 °C) for one week and fed with *Isochrysis galbana*. Then, mussels were divided in 6 glass aquaria (30 L, 30 animals in each tank) and exposed to 0 μg L^{-1} (control group; CTRL; 2 tanks), 0.10 μg L^{-1} Amyl salicylate (low concentration; AS_L; 2 tanks) and 0.50 μg L^{-1} Amyl salicylate (high concentration; AS_H; 2 tanks) and sampled after 7 and 14 days. AS was purchased from Sigma-Aldrich (Milano, Italy; code 44,041, purity ≥98%). The levels chosen for the exposure experiments correspond to median concentrations detected in most impacted Venice lagoon areas (unpublished data). Natural seawater transported to the laboratory for the experimental manipulation was renewed every two days during the experiment.

2.2. Chemical analysis

AS concentrations were measured in AS_L and AS_H water 15 min and 24 h after AS addition. *n*-hexane, dichloromethane, and ultrapure water (10 mL each) were employed to condition the Oasis HLB cartridges 6 cc (200 mg), Waters, Milford, MA, (USA), using then phenanthrene ^{13}C as internal standard and then utilized to extract water samples (0.05 L). The elution of samples was performed using toluene (1 mL), followed by dichloromethane (15 mL) and *n*-hexane (10 mL). Na_2SO_4 was used for eluates drying that were then concentrated to 100 μL under a gentle nitrogen flow at 23 °C (Turbovap II®, Caliper Life Science, Hopkinton, MA, USA).

Bioaccumulation of AS was also determined in pools of mussels (each composed by 5 individuals) from each experimental group at day 7 and 14 of exposure. The pools of the total mussel tissues were homogenized using Ultra-Turrax (IKA) extracted by means of a QuEChERS method. About 3.7 g of wet sample were weighted into a centrifuge tube and spiked using phenanthrene ^{13}C, together with 7 mL of UPW and 10 mL of acetonitrile. After 1 min of vortex, magnesium sulfate (6 g), sodium chloride (1.5 g), sodium citrate dibasic sesquihydrate (0.75 g) and sodium citrate tribasic dihydrate (1.5 g) were added for phase-separation adjustment. Following the vortex performed for 1 min, samples were centrifuged for 5 min at 3000 RPM. Supernatants were transferred to another centrifuge tube contaning 825 mg of Supelclean™ PSA (Primary-Secondary Amine), 2.5 g of magnesium sulfate, 1 g of Na_2SO_4 and 825 mg of Discovery® DSC-18, vortexed for 1 min and centrifuged for 5 min at 3000 RPM. Samples were concentrated to 100 μL, with solvent exchange with dichloromethane. Instrumental analyses were performed by GC-MS/MS (Trace 1310 - TSQ 9000 Thermo Fisher) as described in Picone et al., (2021). Procedural blanks resulted at 0.31 ± 0.07 ng L^{-1} (Method Detection Limit, MDL = 0.21 ng L^{-1}) for water and 0.18 ± 0.06 ng g^{-1} (MDL = 0.19 ng g^{-1}) for biota. Trueness and Relative Standard Deviation of the methods were respectively −9 ± 3% for water and −7 ± 9% for biota analyses.

Furthermore, the Bioaccumulation Factors of AS was calculated to evaluate the uptake in mussels' body as the ratio of tissue and water concentrations (BAF = μg $kg^{-1}_{wet\ weight}$/μg L^{-1}_{water}) following Streets et al., (2006).

2.3. Haemocyte, gill and digestive gland biomarkers

Haemolymph (500 μL per animal at least) was sampled from the anterior adductor muscle by means of a 1 mL-plastic syringe and kept in Eppendorf tubes at 4 °C. For each experimental groups, 5 pools of haemolymph from 4 mussels each were prepared. An aliquot of freshly collected haemolymph was used for measurement of total haemocyte count (THC), haemocyte diameter and volume, lactate dehydrogenase (LDH) activity and haemocyte proliferation (see below for detailed methods). In this regards, pellets (=haemocytes) from centrifuged haemolymph samples (780×*g* for 10 min) were added with an equal volume

of distilled water, vortexed for 30 s and centrifuged at 780×*g* for 10 min at room temperature to obtain haemocyte lysate (HL) samples, whereas supernatants (=cell-free haemolymph, CFH) were collected. Both HL and CFH samples were frozen and stored at −80 °C.

After haemolymph collection, 5 pools of gills and digestive gland from 4 mussels were prepared per each experimental condition and divided in aliquots, which were frozen in liquid nitrogen and stored at −80 °C until analyses.

THC, expressed as the number of haemocytes (10^7)/mL of haemolymph, as well as haemocyte diameter (μm) and volume (picolitres, pL), were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). The *Cell proliferation* Kit II (Roche) was used to measure haemocyte proliferation: 200 μL of the mixture available in the kit was added to 400 μL of haemolymph and incubated for 4 h in a dark humidified chamber. Absorbance values were determined at 450 nm (Beckman 730 spectrophotometer) and results were normalised to THC values of each experimental groups and expressed as optical density (OD_{450}).

The *Cytotoxicity Detection* Kit (Roche) was used to measure CFH lactate dehydrogenase activity (LDH) and the results were expressed as optical density (OD) at 490 nm.

Lysozyme activity was determined mixing 50 μL of HL with 950 μL of a suspension of *Micrococcus lysodeikticus* (0.15% in 66 mM phosphate buffer, pH 6.2). Reduction in absorbance was monitored at 450 nm for 3 min at room temperature, and the results were expressed as μg lysozyme/mg of protein. The method of Bradford (1976) was used to measure total protein concentrations in HL samples.

Gills and digestive gland samples were homogenized at 4 °C with a TissuLyser LT (QIAGEN) in 4 vol of Tris-HCl buffer 10 mM, pH 7.5, containing 0.15 M, 0.5 M and mM of KCl, sucrose, EDTA respectively and protease inhibitor cocktail (Merck). Tissue samples were centrifuged at 12,000 g for 30 min at 4 °C and supernatants (SN) were collected for analyses.

Total superoxide dismutase (SOD) activity was measured in both gills and digestive gland in triplicate using the xanthine oxidase/cytochrome *c* mixture (Crapo et al., 1978). Results are expressed as U/mg protein, one unit of SOD being defined as the amount of SN causing 50% inhibition of cytochrome reduction. Catalase (CAT) activity (U/mg protein, one unit of CAT being defined as the amount of enzyme that causes the dismutation of 1 μmol of H_2O_2/min) was measured at 240 nm in tissue SN samples in triplicate according to Aebi (1984). Glutathione reductase (GR) activity (U/mg protein) was measured following the method of Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. Glutathione S-transferase (GST) activity (nmol/min/mg protein) in the supernatant of digestive gland was evaluated using the procedure described in Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. Acetylcholinesterase and butyrylcholinesterase (AChE and BChE, respectively) activities were evaluated in gill SN according to the procedure of Ellman et al. (1961), using acetylthiocholine or butyrylthiocholine and dithiobisnitrobenzoate as reagents. The results were expressed as nmol/min/mg of protein.

In all assays, total protein concentration of tissue homogenates was measured using the method of Bradford (1976).

2.4. Statistical analysis

As for biomarker results, the normal distribution of data and the homogeneity of the variances were evaluated by the Shapiro-Wilk's test and the Bartlett's test, respectively. The effects of the factors "treatment", "time" and "treatment-time interaction" were assessed by means of a two-way ANOVA (with treatment and time as predictors), whereas pairwise comparisons were performed by Tukey's HSD post-hoc test. All results are expressed as means ± standard error (n = 5).

2.5. Gene expression profiling

RNA was extracted from aliquots of digestive gland from 4 individuals of mussels pooled together, for a total of 5 pools for each experimental group using RNeasy Mini Kit (Qiagen). Further DNAse (Qiagen) treatment was performed. Qubit Fluorometer (Invitrogen) and Bioanalyzer 2100 (Agilent Technologies) were then used to verify the RNA concentration and integrity. Extracted RNA from each pool was then used for both microbiota (16S; described below) and gene expression (RNA-Seq) analyses.

For gene expression analyses, libraries were constructed using 3′ QuantSeq kit (Lexogen) and then sequenced (75 bp SE) on NextSeq 500 Illumina (CRIBI; University of Padova) (BioProject PRJNA793756). Bioinformatics analyses performed the quality of the Illumina raw reads by means the FastQC (v0.11.6) tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Regions discovered being of low quality and adapters were then subjected to the trimming using Trimmomatic (v0.365) (Bolger et al., 2014). The mapping of RNA-seq reads was performed using the *M. galloprovincialis* genome (GeneBank acc. GCA_900618805.1) by means of Rsubread v 2.4.2 software (Liao et al., 2019) using default parameters. Read counts, carried out at gene level, were sorted by *featureCounts* function in Rsubread. Homology relationship between *M. galloprovincialis* and *Crassostrea gigas* was reconstructed with the software OrthoFinder (Emms and Kelly, 2015) that employs the BLAST tool (Altschul et al., 1990) and the MCL algorithm of clusterization (Enright, 2002) to define the orthology groups (OGs). To reach such a goal, the protein dataset referred to *C. gigas* was downloaded from Ensembl public database (Ensembl GCA_000297895.1). For each sample, R v.3.5.3 was used to calculate the sum of raw read counts of all genes belonging to the same OG. OGs with a miscount <3 in more than the 50% of samples of each group were not included in the following analyses. The normalization of remaining OGs (n = 16.358) was performed with RUVs ("k" = 7) within the package RUVSeq/v1.18 (Risso et al., 2014). EdgeR (Robinson et al., 2010) was then employed for the identification of differentially expressed genes (DEGs; FDR ≤0.05). Gene expression was studied through the Principal Component Analysis (PCA) and pairwise comparisons between the CTRL and treated groups (AS_L, AS_H) after both 7 and 14 days of exposure. Furthermore, as follow, in order to study coordinated alterations in the expression of all genes obtained from the pairwise comparison, the Gene Set Enrichment Analysis (GSEA) was performed using the clusterProfiler/v3.12.0 R package (Yu et al., 2012) by means the custom gene sets included in https://www.gsea-msigdb.org/gsea/index.jsp (Subramanian et al., 2005). KEGG pathways and biological processes significantly disrupted following chemical exposures in our previous studies were considered (e.g., Milan et al., 2013; Milan et al. 2015; Milan et al. 2016; Milan et al. 2018; Iori et al., 2020) and FDR <0.25 was set as threshold for gene sets significance. The full list of investigated GO_BP and KP is reported in Supplementary Table 3 and the reference list of pathways in Supplementary File 3.

2.6. Microbiota characterization

Retro-transcription of extracted RNA from pools of digestive gland was carried out using the Superscript IV kit (Invitrogen). Specific reverse and forward primers (V3–V4 gene region of 16 S rRNA) were used for the preparation of libraries, as well as the sequencing of 16S by BMR Genomics (Padua, Italy) as described in Milan et al. (2018) obtaining a total of 4,582,624 reads (BioProject PRJNA793756). The open-source bioinformatics pipeline QIIME2 (Bolyen et al., 2019) was used to perform microbiome analysis from raw sequencing data. The trimming, filtering and merging were conducted with cutadapt and DADA2. Features alignment was carried out with MAFFT (Katoh and Standley, 2013). The output of sequences merging resulted in a total of 1,519,962 reads. Python library scikit-learn and a pre-trained SILVA-database (Yilmaz et al., 2014) were employed for the classification of merged

reads and for taxa assignment. The outputs, including the feature table and taxonomy, were used to perform the Principal Coordinates Analysis (PCoA) and the pairwise comparison analyses between the CTRL and the treated groups (AS_L, AS_H) carried out by means the one-way ANOVA at species and genus levels (p-adj<0.05) by CALYPSO online software (v.8.84). In addition, species richness and equitability were determined by Evenness and Simpson's index.

3. Results

3.1. Chemical analyses

Results of chemical analyses are reported in Supplementary File 1. During lab exposure, AS concentrations detected in AS_L and AS_H tanks after 15 min from AS application were 49–85 10^{-3} μg L^{-1} and 276–565 10^{-3} μg L^{-1}, respectively. However, 24 h after AS addiction, concentrations dropped to levels comparable to control tanks (Supplementary file 1). Concerning bioaccumulation, AS concentrations in CTRL mussels ranged between 2.2 μg kg^{-1} and 2.3 μg kg^{-1}, indicating previous exposures to AS in Venice lagoon. Mussels exposed to 0.1 μg L^{-1}, showed AS concentrations of 2.9 μg kg^{-1} and 3.2 μg kg^{-1} at day 7 and 14, respectively. As expected, the highest bioaccumulation was detected in individuals exposed to 0.5 μg L^{-1}, resulting in 6.9 μg kg^{-1} at day 7 and 5.9 μg kg^{-1} at the last sampling time. The BAF values obtained were 29 (day 7) and 32 (day 14) in AS_L exposed mussels, while following AS_H exposures BAF were 14 and 12 at day 7 and day 14, respectively.

3.2. Haemocyte, gill and digestive gland biomarkers

Two-way ANOVA analysis revealed a significant ($F_{2,24} = 704.77$; $p < 0.001$) effect of treatment and treatment-time interaction ($F_{2,24} = 22.60$; $p < 0.001$) on THC values of mussels, with a significant ($p < 0.001$) decrease in THC after 7 days of exposure to AS_H (Fig. 1A). Exposure of mussels to AS significantly affected the volume ($F_{2,24} = 4.46$; $p < 0.05$) and diameter ($F_{2,24} = 6.33$; $p < 0.01$) of haemocytes ($p < 0.05$), with a significant ($p < 0.01$) increase after 7 days in AS_H-exposed mussels (Fig. 1B and C).

Exposure time ($F_{1,24} = 7.05$; $p < 0.05$), treatment ($F_{2,24} = 13.26$; $p < 0.001$) and the interaction between time and treatment ($F_{2,24} = 10.15$; $p < 0.001$) significantly influenced haemocyte proliferation. In details, pairwise comparisons highlighted a significant increase in cell proliferation in mussels treated for 7 days with AS_H ($p < 0.001$; Fig. 1D). Conversely, none of the experimental factors affected LDH activity (data not shown). The factors time ($F_{1,24} = 9.51$; $p < 0.01$), treatment ($F_{2,24} = 7.22$; $p < 0.01$) and time-treatment interaction ($F_{2,24} = 3.85$; $p < 0.05$) also influenced HL lysozyme activity, that increased in mussels exposed for 14 days at AS_H (Fig. 1E).

Exposure to AS did not significantly ($F_{2,24} = 1.27$; $p > 0.05$) influence gill SOD activity, while only exposure time influenced enzymatic activity in digestive gland ($F_{1,24} = 25.96$; $p < 0.001$) (Fig. 2A and B). Exposure time ($F_{1,24} = 10.22$; $p < 0.01$), treatment ($F_{2,24} = 4.04$; $p < 0.05$) and time-treatment interaction ($F_{2,24} = 5.14$; $p < 0.05$) affected CAT activity of digestive gland. Pairwise comparisons revealed that at day 7 digestive gland CAT activity significantly increased in AS_L and decreased in AS_H, with respect to controls (Fig. 2C and D). Exposure time ($F_{1,24} = 7.49$; $p < 0.05$) and time-treatment interaction ($F_{2,24} = 4.98$; $p < 0.05$) significantly influenced gill CAT activity, with a significant reduction in bivalves treated for 14 days with both AS concentrations ($p < 0.05$ and $p < 0.01$, respectively). None of the experimental factors (time: $F_{1,24} = 2.082$, $p > 0.05$; treatment: $F_{2,24} = 1.23$, $p > 0.05$; time-treatment interaction: $F_{2,24} = 1.09$, $p > 0.05$) significantly influenced GST activity, while only exposure time significantly affected both AChE ($F_{1,24} = 22.17$, $p < 0.001$) and BChE ($F_{1,24} = 39.80$, $p < 0.001$) in gills (data not shown).

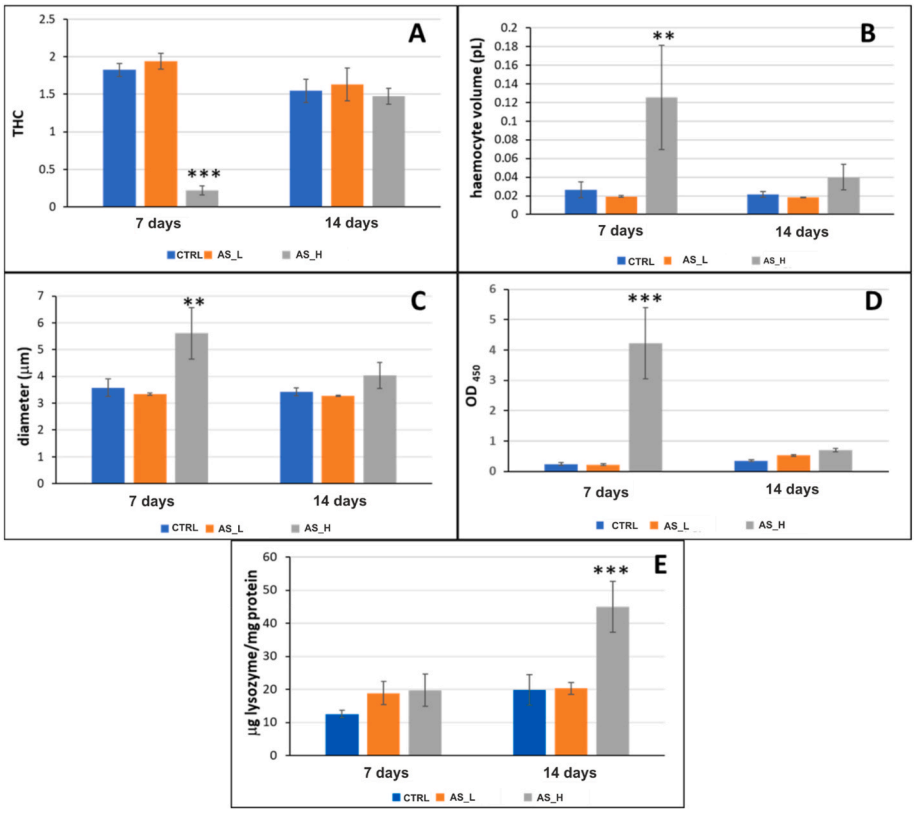

Fig. 1. A) Total haemocyte count (THC), B) haemocyte volume, C) haemocyte diameter, D) cellular proliferation and E) lysozyme activity. In all graphs, asterisks indicating statistically significant variations between treated groups and the related controls at day 7 and 14 were showed in case of significant effects of the factors "treatment" and/or "treatment-time interaction" (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

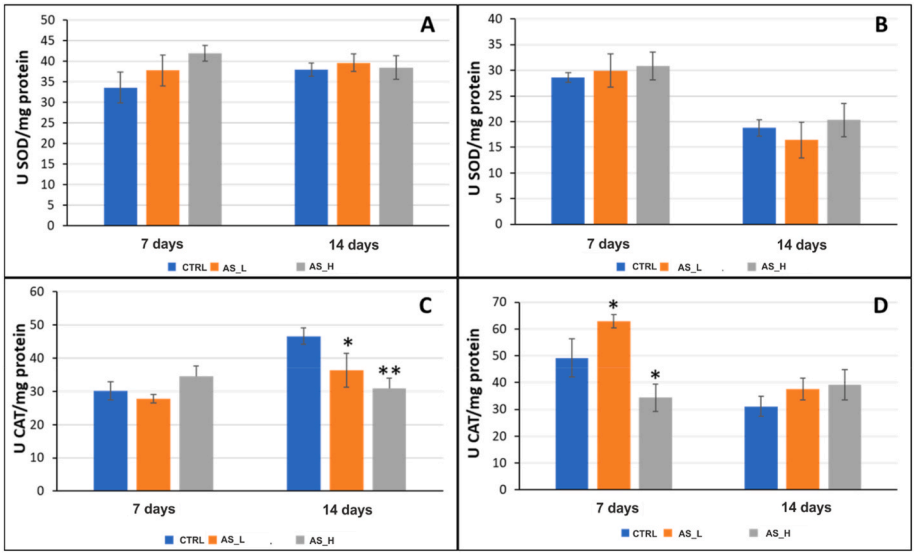

Fig. 2. A) SOD activity in gills, B) SOD activity in digestive gland, C) CAT activity in gill, D) CAT activity in digestive gland. In all graphs, asterisks indicate significant variations between treated groups and the related controls at day 7 and 14 (*$p < 0.05$, **$p < 0.01$).

3.3. Microbiota characterization

PCoA analysis highlighted the separation along the y-axis between mussels collected at the two sampling points (PC2 = 17%) (Fig. 3A), while a weak separation of AS_H from other treatments has been highlighted along the y-axis after 14 days of AS exposure (PC2 = 23%) (Fig. 3B and C). No significant changes in microbial diversity (i.e., Simpson's Index) and Evenness have been observed in exposed mussels compared to controls (Fig. 3D and E).

Pairwise comparisons between CTRL and exposed mussels allowed the identification of differentially represented taxa in exposed mussels compared to controls (p-adj value < 0.05; Table 1). Both treatments showed few significant changes in microbial composition, mostly at day 7. Among them, over-representation of *Vibrio aestuarianus* and *Arcobacter* genus was observed in mussels of AS_L treatment. Conversely, all significant taxa identified in AS_H exposed mussels were down-represented compared to controls at day 7, while, no modifications in microbiota composition were observed at the last sampling time.

3.4. Gene expression profiling

Like microbiota analyses, PCA revealed a clear separation between mussels collected at day 7 and 14 along the X-axis (PC1 = 9.9%; Fig. 4A). While at day 7 weak separations among treatments were highlighted (Fig. 4B), at day 14 AS_H exposed mussels were clearly separated from other groups along X- and Y-axis (PC1 = 9.9%; PC2 = 6.7%) (Fig. 4C). The number of DEGs obtained by pairwise comparisons between exposed and control groups reflects the results obtained by PCA, with the highest number of DEGs found in AS_H exposed mussels at day 14 (68 DEGs; Table 2). Conversely, AS_L exposed mussels showed a total of 23 and 14 DEGs at day 7 and 14, respectively. At day 7, the highest number of DEGs was down-regulated in AS exposed mussels (68% and 78% in AS_H and AS_L, respectively). None of the DEGs in response to AS_L were maintained differentially expressed at day 7 and 14, while a unique transcript coding for *Protocadherin-15* was down-regulated at both sampling points in AS_H. The full lists of DEGs obtained by pairwise comparisons at each sampling time are reported in Supplementary File 2.

Focusing on the transcriptional response at day 7, mussels exposed to AS_L showed the down-regulation of genes coding for *N66 matrix protein* (*N66*), *Gigasin-6*, *Heme-binding protein 2* (*HEBP2*), Serpin B3 (*SERPB3*) and *Mytilin-3* (*MYT3*), while a down-regulation of *Heme-binding protein 1* (*HEBP1*) was observed in AS_H. At day 14, AS_L showed the up-regulation of several extracellular matrix structural constituents playing a role in modulation of cell adhesion, as *Collagen alpha-5(VI) chain* (*COL6A5*), *Collagen alpha-2(XI) chain* (*COL11A2*) and *transforming growth factor-beta-induced protein (TGFBI)*. In addition, up-regulation of *Cyclic AMP-dependent transcription factor* (*ATF-3*) and *Mucin-12* (*MUC12*), a protein forming protective mucous barriers on epithelial surfaces, has been also observed. Noteworthy, after 14 days *Heat shock* 70 kDa *protein 12B (HSPA12B)* and *Tripartite motif-containing protein 45* (*TRIM45*) were differentially expressed in both treatments. In addition, mussels exposed to AS_H showed the up-regulation of *Histone deacetylase 6* (*HD6*) and *Histone-lysine N-methyltransferase* (*SETD8*) and the down-regulation of *TLR4 interactor with leucine rich repeats* (*TRIL*), *Toll-like receptor 13* (*TLR13*), *Poly [ADP-ribose] polymerase 14* (*PARP14*), *Apoptosis inhibitor 5* (*API5*) and *Solute carrier family 6 member* (*SLC6*).

As expected, GSEA showed the highest number of altered pathways in AS_H treatment at both sampling times (FDR<0.25; Table 3). At day 7, just two KEGG pathways representing "apoptosis" and "NOD-like receptor signaling pathway" were significantly enriched in AS_L exposed mussels, while AS_H exposed mussels showed the up-regulation of "autophagic cell death" (FDR<0.1) and several pathways involved in energy metabolism, xenobiotic metabolism, and stress response including "lysosome", "proteasome" and "PPAR signaling pathway".

At day 14, GSEA indicated an increased number of significantly enriched pathways in AS_L mussels, including the up-regulation of "lysosome", "proteolysis" and "drug metabolism cytochrome p450" among others. At the same sampling time, in addition to pathways related to energy metabolism, xenobiotic metabolism, "autophagic cell death" and "PPAR signaling pathways" already detected at day 7, AS_H exposed mussels showed the up-regulation of the KEGG pathway "pathways in cancer" (FDR≤ 0.15), and of the GO terms "cellular response to external stimulus" and "glutamatergic synapse". To conclude, following AS_H exposures, the up-regulation of the immune terms "toll-like receptor signaling pathways", "response to virus" and "immune system process" was also observed. Results of GSEA analyses are reported in Supplementary File 3 and summarized in Table 3.

4. Discussion

4.1. Amyl salicylate bioaccumulation capability

While Amyl Salicylate has been widely detected in different aquatic

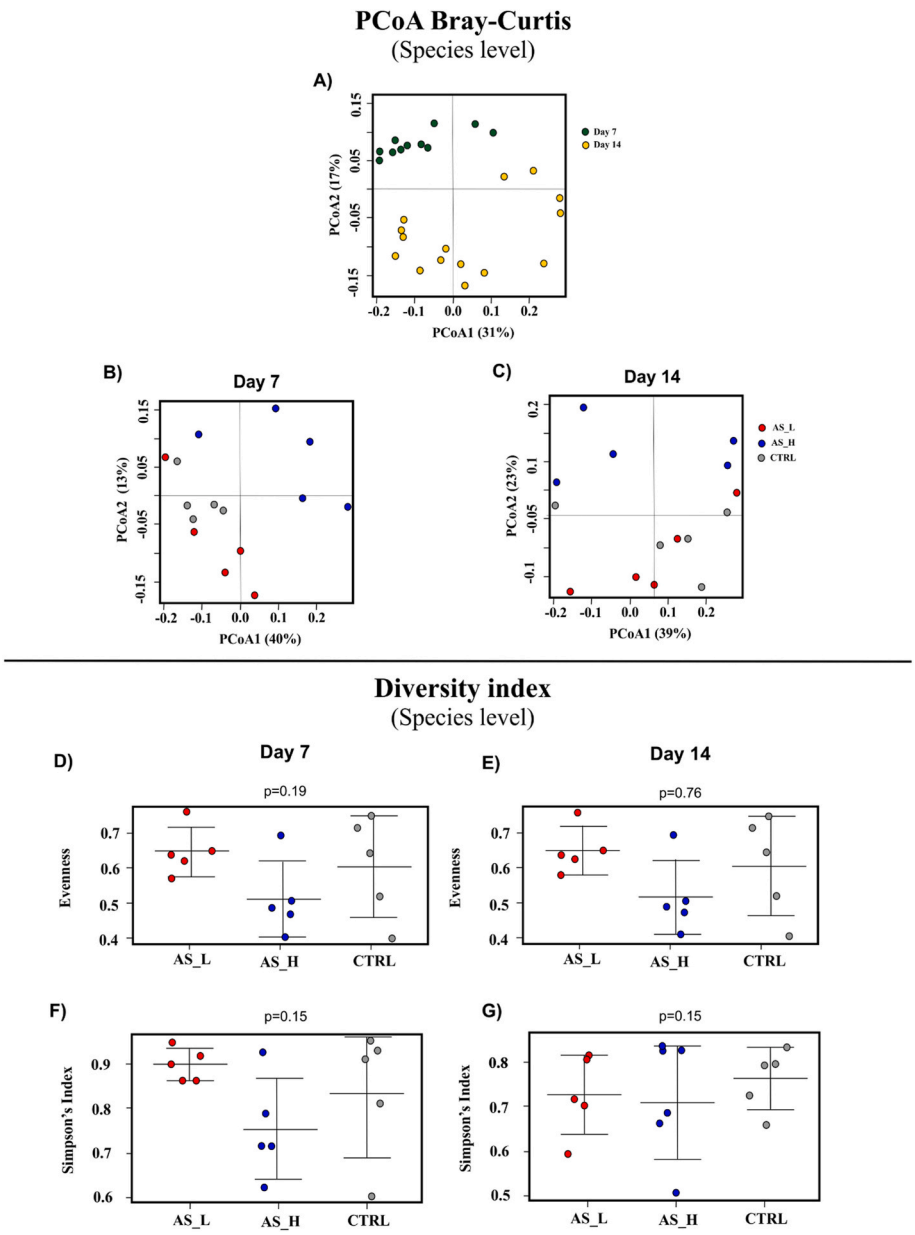

Fig. 3. Principal Coordinates Analysis (PCoA) of mussel digestive gland microbiota. A) PCoA of mussels' digestive gland collected at 7 and 14 days of exposure; different colours indicate the two sampling time (Day 7 and Day 14); B) PCoA of mussels digestive gland collected after 7 days of exposure; different colours indicate different treatment at each sampling time. C) PCoA of mussel's digestive gland collected after 14 days of exposure; different colours indicate different treatment at each sampling time. D) Evenness index for each treatment after 7 days of exposure. E) Evenness index for each treatment after 14 days of exposure. F) Richness diversity index (Simpson's Index) for each group at day 7 of exposure. G) Richness diversity index (Simpson's Index) for each group at day 14 of exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

environments worldwide, data about its bioaccumulation are still scarce. Here, AS bioaccumulation was investigated for the first time in a mussel species following exposures to concentrations that resemble the environmental levels found in farming areas of the Venice lagoon. First, it should be highlighted that chemical analyses showed a fast reduction of AS concentrations in seawater used during controlled exposures that dropped to levels comparable to the control after 24 h. This result suggests a relatively fast kinetics of adsorption/removal of AS in aqueous solution. Concerning bioaccumulation, while mussels exposed to the lower AS concentrations showed a weak increase of AS in tissues, concentrations three times higher than the control group was observed in AS_H mussels. Noteworthy, despite mussels were maintained for 14 days in laboratory conditions, AS was also detected in mussels from the control group, confirming the spread of this fragrance in the Venice lagoon and indicating its bioaccumulation also in the natural environment. However, being chemicals considered to be bioaccumulative for aquatic organisms with BAFs exceeding 5000, AS showed only a mild tendency to bioaccumulation for *M. galloprovincialis*.

4.2. Cellular, biochemical and molecular analyses revealed potential AS immunotoxicity

Cellular and biochemical analyses here performed indicate that mussels exposed to higher AS concentrations showed significant and rapid alterations in THC and haemocytes diameter, volume and proliferation. Similar results were recently reported in other studies assessing the effects of PCPs. For example, it has been demonstrated that the fragrance Galaxolide can affect THC and haemocyte proliferation in the clam *Ruditapes philippinarum* (Rilievo et al., 2021). In addition, decreased THC and haemocytes alterations were observed in mussels treated with a mixture of contaminants, including herbicide, synthetic estrogen and Amyl salicylate (Fabrello et al., 2021), as well as in clams exposed to Triclosan (Matozzo et al., 2012a), an antibacterial agent included in the PCPs list.

Overall, the alteration of the number, diameter, volume and proliferation of haemocytes, being the defense line against different stressors (Mayrand et al., 2005; Zannella et al., 2017), suggests potential ability of AS to trigger disorders in the mussel's immune system. Interestingly, in

Table 1
Lists of significant genera and species obtained comparing control and exposed mussels AS_L and AS_H after 7 and 14 days of exposure (Adjusted p-value < 0.05). Down- and up-represented genera/species in exposed mussels are reported in green and red respectively.

Significant taxa (p-adj.<0.05)			
Genus level			
AS_L		AS_H	
Day 7	Day 14	Day 7	Day 14
uncultured_Grampositive_bacterium	Glaciecola	Hyphomonas	
Arcobacter	Coxiella	Spongiibacter	
Spongiibacter		Zhongshania	
		Pseudofulvibacter	
Species level			
AS_L		AS_H	
Day 7	Day 14	Day 7	Day 14
Vibrio_Vibrio_aestuarianus	Glaciecola_uncultured_gamma_proteobacterium_CHABXII8	Hyphomonas_uncultured_bacterium	
Spongiibacter_Spongiibacter_sp._IMCC21906		Spongiibacter_Spongiibacter_sp._IMCC21906	
Neptuniibacter_Raricirrus_beryli_associated_bacterium		Zhongshania_uncultured_bacterium	
		Pseudofulvibacter_uncultured_bacterium	

this study increased haemocyte proliferation was observed in mussels treated for 7 days with AS_L, while THC decreased dramatically in the same animals. Probably, those mussels underwent an increase in cell proliferation to balance, at least in part, the marked decreases in the number of circulating cells. Similarly, an inverse relationship between THC and haemocyte proliferation was found in *R. philippinarum* exposed to galaxolide (Rilievo et al., 2021) and ibuprofen (Matozzo et al., 2012b).

Potential disruption in the regulation of the immune system has also been suggested by gene expression profiling. In particular, mussels exposed to AS_H showed the up-regulation of the immune terms "toll-like receptor signaling pathways", "defense response to virus" and "immune system process" as well as the down-regulation of *TRIL* and *TLR13,* both involved in inflammatory/immune responses. Similarly, mussels exposed to AS_L showed the down-regulation at day 7 of *SERPB3* belonging to a large protein family known to regulate innate immunity pathways, and *Mytilin-3* (*MYT3*), a well-known anti-microbial peptide in mussel species (Mitta et al., 2000).

As already observed in previous studies, immune system alterations may also lead to reduced ability to face modifications in microbiota community and opportunistic pathogens spread following environmental stress (Burgos-Aceves and Faggio, 2017; Zannella et al., 2017; Iori et al., 2020). Accordingly, microbiota characterization assumes a key role to establish possible host-microbiota interactions and to investigate chemicals indirect effects on mussel health related to changes in their microbial composition.

As for antioxidant enzymes, this study demonstrated that AS affected only CAT activity in both gills and digestive gland. However, a different response between such tissues was recorded. Indeed, CAT activity reduced in gills of mussels treated for 14 days to both AS concentrations tested, whereas enzyme activity increased in digestive gland of bivalves exposed for 7 days to AS_L, but decreased in mussels treated with AS_H. The non-linear pattern of variation of SOD and CAT in the two tissues was also recorded in clams exposed to galaxolide (Rilievo et al., 2021). Summarizing, results of the present study suggest that AS is not an oxidative stress promoter, at least under the investigated experimental conditions and in the mussel species tested. However, other studies are needed to fully understand the mechanism of action of AS on antioxidant enzymes, including those not measured in this study.

4.3. *Microbiota analyses showed transient spread of opportunistic pathogens at low AS concentrations*

The study of microbiota community in marine benthic filter-feeder organisms is acquiring increasing importance due to its beneficial role in the protection against pathogens and environmental stressors (e.g., Lokmer and Wegner, 2015; Meisterhans et al., 2016; Milan et al., 2018). On the other hand, following the exposure to environmental stress, opportunistic taxa may take advantage of the compromised host physiological status, playing a key role in shellfish mortality events and stocks reductions (Milan et al., 2019; Destoumieux-Garzón et al., 2020; Iori et al., 2020; Bernardini et al., 2021; Mathai et al., 2021; Richard et al., 2021).

Our study indicates that exposure to AS lead to weak and transient changes in the microbiota of the digestive gland. Unexpectedly, the most important microbial changes have been observed in mussels exposed to AS_L at day 7, with the increased representation of the opportunistic pathogens *Arcobacter* and *Vibrio aestuarianus*, two Gram-negative

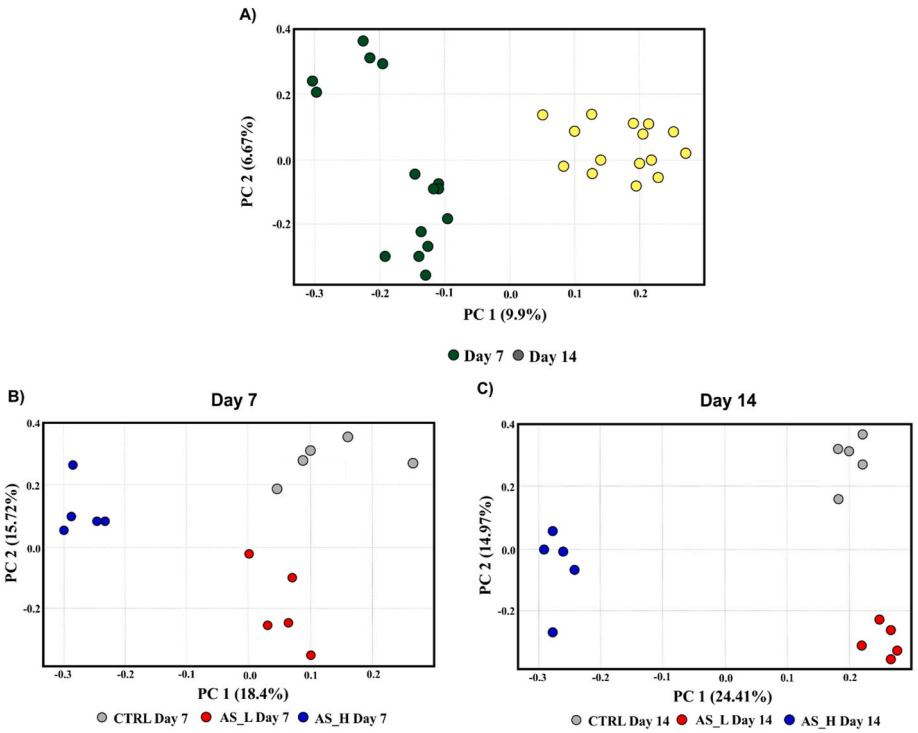

Fig. 4. PCA applied to gene expression profiles of mussels exposed to different Amyl salicylate concentrations (AS_L = 0.1 μg L^{-1}; AS_H = 0.5 μg L^{-1}). A) PCA performed considering all samples. Different colours indicate sampling times. B) and C) showed PCA performed at day 7 and 14, respectively. Treatments are indicated with different colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Number of total, down- (↓) and up- (↑) differentially expressed genes (DEGs) (FDR ≤0.05) for each pairwise comparison after 7 and 14 days of AS exposure.

	AS_L			AS_H		
	N° total DEGs	N° ↑	N° ↓	N° total DEGs	N° ↑	N° ↓
Day 7	**23**	5	18	**16**	5	11
Day 14	**14**	2	12	**68**	31	37

bacteria widely described in marine environment and in bivalves exposed to stressful environmental conditions (Beaz-Hidalgo et al., 2010; Vezzulli et al., 2010; Bernardini et al., 2021; Iori et al., 2020; Milan et al., 2018). *Arcobacter* was frequently reported as one of the dominant taxa in unhealthy or moribund marine animals, (Tanaka et al., 2004; Fan et al., 2013; Olson et al., 2014; Lokmer and Wegner, 2015). Similarly, *Vibrio aestuarianus* is a well-known shellfish pathogen (Tison and Seidler, 1983) able to impair haemocytes functional response in *Crassostrea gigas* (Labreuche et al., 2006, 2010) and associated to mussels and oyster mortality events (Saulnier et al., 2009; Romero et al., 2014). However, the over-representation of these pathogens in exposed mussels occurs just in the early exposure phase, while at day 14 none of these taxa were over-represented in AS exposed mussels. Overall, our results indicated that AS lead to weak changes in digestive gland microbiota and that mussels can rapidly restore the "normal" microbial communities, as suggested also by the lack of differentially represented taxa in mussels exposed to AS_H at day 14. To our knowledge, this study represents the first attempt to describe microbial changes occurring in marine species following the exposure to fragrance compounds.

4.4. Early transcriptional changes following amyl salicylate exposures

After 7 days of AS exposures both experimental groups showed the down-regulation of the majority of DEGs. A possible explanation of this result is a process called "Stress-Induced Transcriptional Attenuation (SITA)", consisting in rapid transcriptional down-regulation to protect cells from damage following environmental stress (Aprile-Garcia et al., 2019). However, it should be also noted that GSEA highlighted the up-regulation in AS_H exposed mussels of several molecular pathways involved in drug metabolism and stress response. Among them, the up-regulation of the molecular pathways "Drug metabolism cytochrome P450", and "xenobiotic metabolism" indicates the rapid activation of detoxification processes. In addition, the up-regulation of molecular pathways such as "ion transmembrane transport", "golgi apparatus", "lysosome", "PPAR signaling pathway" (maintained also at day 14), "proteasome" and "arachichidonic acid metabolism" suggests that exposure to AS_H lead to the rapid activation of molecular pathways involved in stress response to face possible dysfunctions at cellular level. Conversely, GSEA suggested weak transcriptional changes in mussels exposed to the low AS concentration, showing just the up-regulation of "apoptosis" and "NOD-like receptor signaling pathway". These molecular pathways, involved in the regulation host innate immune response (Carneiro et al., 2004; Zhang et al., 2015; Jiang et al., 2020), have been already found up-regulated in bivalve species following *Vibrio* spp. infections. (Ren et al., 2017, 2020; Wang et al., 2019; Zuo et al., 2020) suggesting potential links with the spread of *Vibrio* spp. and *Arcobacter* here described in the same individuals.

At the early stage of AS exposures, transcriptional changes in Heme-binding proteins *HEBP1* and *HEBP2* have been also observed following exposures to both AS concentrations. Disruptions in transcriptional regulation of heme binding genes were described in invertebrate species exposed to arsenic and copper (Moreira et al., 2018; Ki et al., 2009), as well as in *Ostreid herpesvirus-1* infected oysters (He et al., 2015). While their specific role in bivalve species still needs to be elucidated, possible biological functions of these proteins in oxidative stress response, apoptosis and immune response have been recently proposed (Fortunato et al., 2016).

To conclude, exposure to AS_L concentration led also to the down-

Table 3
Gene Set Enrichment Analysis: list of molecular pathways significantly down- (in green) and up- (in red) regulated in each treatment/sampling time (FDR<0.25). Full list of investigated pathways is reported in Supplementary File 3.

	AS_L Day7	AS_H Day7	AS_L Day14	AS_H Day14
Energy Metabolism				
Atp biosynthetic process				
Oxidative phosphorylation				
Citrate cycle tca cycle				
Fatty acid metabolism				
Glycolysis-gluconeogenesis				
Xenobiotic Metabolism				
Xenobiotic metabolism				
ABC transporters				
Drug metabolism cytochrome p450				
Stress Response				
Pathways in cancer				
Cellular response to external stimulus				
Ion transmembrane transport				
Transport vesicle				
Golgi apparatus				
Focal adhesion				
Lysosome				
PPAR signaling pathway				
Proteasome				
Proteolysis				
Neurotransmisison/Synapse				
Glutamatergic synapse				
Apoptosis/Cell Death				
Autophagic cell death				
Regulation of cell death				
Apoptosis				
Signalling				
NOD like receptor signaling pathway				
p53 signaling pathway				
Toll like receptor signaling pathway				
Immune response/Inflammation				
Defense response to virus				
Immune system process				
Arachidonic acid metabolism				

regulation of genes involved in shell calcification, as *N66 matrix protein* (represented by two contigs) and *Gigasin-6* (Rivera-Perez et al., 2019; Santos et al., 2021). The former is a functional shell matrix protein with a key role in biomineralization, widely described in *Pinctada fucata* and in a variety of other diverse mollusk taxa (Kono et al., 2000; Smith--Keune and Jerry, 2009). Over the decades, several studies testified possible effects of a variety of contaminants on shell calcification including the down-regulation of genes involved in shell formation in *M. galloprovincialis* embryos (Alzieu, 2000; Miglioli et al., 2021). Despite further experiments are required, our study provided preliminary indications about potential alterations in biomineralization following exposure to environmental AS concentrations.

4.5. Gene expression analyses suggest detrimental effects following prolonged exposures to amyl salicylate

At the last sampling time, transcriptional changes of *HSPA12B* and *TRIM45* have been observed in both AS exposed group. The former is a member of HSP70 protein family widely described in bivalve species for their role in the response to different stressors (e.g. Cruz-Rodríguez and Chu, 2002; Izagirre et al., 2014; Mezzelani et al., 2021; Song et al., 2006), while the latter belongs to the TRIM family proteins involved in several biological processes, such as cell differentiation and apoptosis (Ozato et al., 2008). *TRIM45* may assume the role of transcriptional repressor of the mitogen-activated protein kinase (MAPK) signaling pathway (Wang et al., 2004). Additional evidence of changes in genes involved in transcriptional regulation have been suggested by the

up-regulation *Histone deacetylase 6*, *SETD8* and *ATF-3*. *SETD8* is a lysine methyltransferase playing primary roles in the regulation of chromatin structure and gene transcription as well as in cellular signal transduction pathways (Greer and Shi, 2012; Black et al., 2012; Biggar and Li, 2015). This protein was found to be conserved in fish and mammals and recently a potential role in the balance maintaining between the internal and external environments has been proposed in *Crassostrea gigas* (Thompson, 2011; Zhao et al., 2016). *ATF-3*, up-regulated in AS_L exposed mussels, is part of ATF/cyclic AMP response element-binding (ATF/CREB), a family of transcription factors involved in apoptosis and cell cycle regulation. Low trascriptional levels are reported in quiescent cells while it is modulated by a variety of signals as cytokines, genotoxic agents, or physiological stresses (Hai et al., 1999; Hall et al., 2020; Thompson et al., 2009). In particular, a relevant role in cellular response to oxidative stress has been proposed and up-regulation has been already described in mussel gills following copper exposures (Hai et al., 1999; Hall et al., 2020).

However, the most important effects at molecular level were observed in mussels treated with the high AS concentration. In particular, changes in transcriptional regulation of *PARP14* and *API5* and the up-regulation of "regulation of cell death" and "autophagic cell death" pathways suggest the disruption of apoptosis and cell death regulations (Soldani et al., 2001; Morris et al., 2006). *API5* acts as an endogenous inhibitor of Caspase-2 and as suppressor of the transcription factor E2F1-induced apoptosis and other nuclear factors involved in apoptotic DNA fragmentation (Morris et al., 2006; Rigou et al., 2009; Imre et al., 2017). PARP14 belong to PARP protein family coding for an anti-apoptotic protein involved also in cell stress responses (Vyas et al., 2014) recently found to be responsive in mussels exposed to glyphosate and AMPA (Iori et al., 2020).

Detrimental effects following prolonged exposure to high AS concentration are also suggested by the down-regulation of "p53 signaling pathway" and the up-regulation of "pathways in cancer" in AS_H exposed mussels. Conversely, the low number of DEGs and the up-regulation of few pathways involved in detoxification and stress response (i.e. "drug metabolism CYP450", "Lysosome", "focal adhesion" and "proteolysis") suggest that chronic exposure to low AS concentrations led to mild conditions of cellular stress.

To conclude, high AS concentrations led also to transcriptional changes in "glutamatergic synapse pathway" and in *SLC6* acting as specific transporters for neurotransmitters, amino acids and oligopeptides. This result is in contrast with results obtained by Rilievo et al. (2021) in Manila clam exposed to the musk fragrance Galaxolide in which no neurotoxic effect has been described. Nevertheless, neurotoxicity of PCPs and fragrances has not been well studied yet, and a more in-depth analysis is required in order to investigate potential neuro-consequences of fragrances on marine organisms.

5. Conclusion

The Mediterranean mussel *M. galloprovincialis* is among the most important economic bivalve species in Italy widely farmed within the Venice lagoon, a representative case of an ecosystem severely impacted by chemicals including emerging contaminants due to urban centres and industrial and agricultural activities. Being a sessile organism, mussels can be subject to chronic or acute exposures to anthropogenic compounds. In our study, we applied a multidisciplinary approach to investigate for the first time the potential effects of Amyl salicylate, one of the most represented emerging contaminants detected in the Venice lagoon.

Chemical analyses suggested mild/low tendency for bioaccumulation of this fragrance in *M. galloprovincialis*. However, the occurrence in the unexposed individuals (control group) indicates potential AS bioaccumulation also in mussels inhabiting clean farming areas, while future studies are needed to investigate bioaccumulation in species/populations inhabiting more impacted Venice lagoon areas, where concentrations up to 6 μg/L-1 were recently detected (unpublished data).

Molecular and cellular analyses suggest that realistic AS concentrations of the Venice lagoon may exert important effects after prolonged exposure periods. While mussels exposed to low AS concentrations showed weak transcriptional changes and transient changes in microbial communities, the exposure to the high AS concentration led to detrimental effects at molecular and cellular levels, including possible changes in immune response, apoptosis and cell death regulations.

To our knowledge, the present study represents the first investigation about the possible ecotoxicity of AS on edible marine filter-feeder bivalves. The multidisciplinary approach here applied allowed to depict the health status and the responses of this marine species, suggesting its application in future studies aiming to define the risk of emerging contaminants on non-target edible species.

Data accessibility

Files of all sequencing are available in NCBI Sequence Read Archive: BioProject PRJNA793756.

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Author statement

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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